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Abstract:	Dinuclear CuII patellamide complexes (patellamides are cyclic pseudo-octapeptides) are known to be efficient catalysts for hydrolysis reactions of biological importance, e.g. phosphatase, carbonic anhydrase and glycosidase. However, the biological role of patellamides is still not known. In a further step to unravel the metabolic significance of the patellamide complexes, the question as to whether these are also formed inside Prochloron cells is addressed. In this study a bio-compatible patellamide-fluorescent-dye conjugate has been introduced in living Prochloron cells, and with flow cytometry and confocal microscopy it is shown that CuII ions are coordinated to patellamides in vivo.
Author Comments:	Dear Editor We have uploaded the manuscript "Is CuII coordinated to patellamides inside Prochloron cells?" by P. Comba, A. Eisenschmidt, L. Gahan, D. Herten, G. Nette, G. Schenk and M. Seefeld, for publication as a full paper in Chem. Eur. J. Patellamides are naturally occurring cyclic peptides that have been shown by us to form dinuclear CuII complexes, which are very active catalysts for phosphoester hydrolysis, carbonate hydration and other biologically important hydrolysis reactions.

	Part of this work has been published in your Journal. For example, if this were the biological function, these "enzymes" would be the first CuII-based carboanhydrases. We therefore now present our first biochemical results of these interesting molecules, and show that CuII indeed is coordinated in living cells of the host species. We hope that your reviewers find our results and interpretations of interest and agree to have this work published in Chem. Eur. J. All authors have seen and approved the manuscript uploaded, and this has not been submitted to any other journal. We are looking forward to your decision. Best regards Peter Comba
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Do you or any of your co-authors have a conflict of interest to declare?	No. The authors declare no conflict of interest.
Animal/tissue experiments?	No

Is Cu^{II} coordinated to patellamides inside *Prochloron* cells?*

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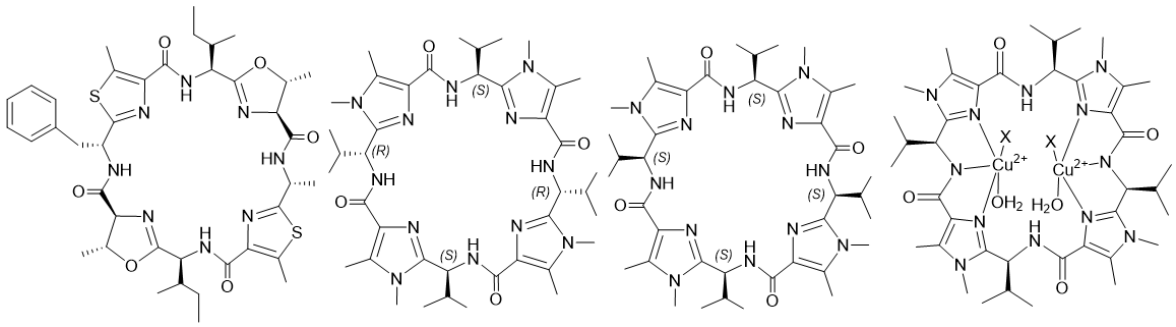
Abstract

Dinuclear Cu^{II} patellamide complexes (patellamides are cyclic *pseudo*-octapeptides) are known to be efficient catalysts for hydrolysis reactions of biological importance, *e.g*. phosphatase, carbonic anhydrase and glycosidase. However, the biological role of patellamides is still not known. In a further step to unravel the metabolic significance of the patellamide complexes, the question as to whether these are also formed inside *Prochloron* cells is addressed. In this study a bio-compatible patellamide-fluorescent-dye conjugate has been introduced in living *Prochloron* cells, and with flow cytometry and confocal microscopy it is shown that Cu^{II} ions are coordinated to patellamides *in vivo*.

Introduction

Small cyclic octapeptides extracted from the blue-green algae *Prochloron* have been the subject of intensive investigations regarding their putative biological role.[1-3] They are commonly referred to as "patellamides", as they were initially isolated from the sea squirt *Lissoclinum patella*, the host of *Prochloron*.[4] Despite its biotoxicity, the concentration of Cu^{II} is significantly increased in the sea squirt *L. patella* when compared to the surrounding sea water,[5] and this has led to a detailed investigation of the metal ion binding behavior of naturally occurring cyclic peptides like ascidiacyclamide and patellamides A-D (Scheme 1).[6] It was shown that the amide nitrogen atoms as well as the azole nitrogens serve as binding sites for Cu^{II} complexation, with two azole-amide-azole binding sites per peptide. To enable the investigation of *in vitro* coordination chemistry and also to make patellamide derivatives available for structure-activity relationships, synthetic routes have been developed to produce cyclic *pseudo*-peptides, delivering various model peptides as an alternative to the biosynthesis and isolation from natural sources (Scheme 1).[2, 7] Detailed investigations on the dinuclear Cu^{II} complexes were carried out in combination with binding affinity measurements demonstrating that the model ligands exhibit similar affinities as reported for the natural patellamides.[2, 8-10]



Scheme 1. From left to right: Patellamide D, model patellamides H_4pat^1 and H_4pat^2, dinuclear Cu^{II} complex of H_4pat^2; X=substrate / H_2O / OH^-.

To shed light on the biological role of the patellamides, hydrolase studies were conducted showing that the dinuclear Cu^{II} complexes of the synthetic peptides act as potent phosphatase and carbonic anhydrase mimics at pH 7-8.[11, 12] At pH 10-11, α- and β-glycosidase as well as β-lactamase activity was observed,[1] giving rise to the idea that metal complexes of the cyclic peptides might act as pH-dependent catalysts. This is underpinned by a study which showed that the pH in *L. patella* varies greatly from pH 7.5 in darkness to almost 11 in the light.[13] Thus, all hydrolase activities observed to-date might be equally metabolically important for *Prochloron*, and the dinuclear Cu^{II} complexes might switch their activity from carbon-assimilation to carbohydrate degradation depending on the level of irradiation. This pH-dependent function is known for metalloproteins as exemplified by the

organophosphate-degrading hydrolase from *Agrobacterium radiobacter*[14] and purple acid phosphatases.[15]

As a first step towards *in vivo* testing of the activities of Cu^{II} patellamide complexes with the ultimate goal being the establishment of the true biological function(s) of the cyclic peptides, we now present our results that answer the question asked in the title: is Cu^{II} coordinated to these ligands in cells? Therefore, the fluorescence and fluorescence quenching by Cu^{II} coordination to a model patellamide-dye conjugate, H_4pat-Atto550, introduced into living *Prochloron* cells, was analyzed by confocal microscopy and flow cytometry.

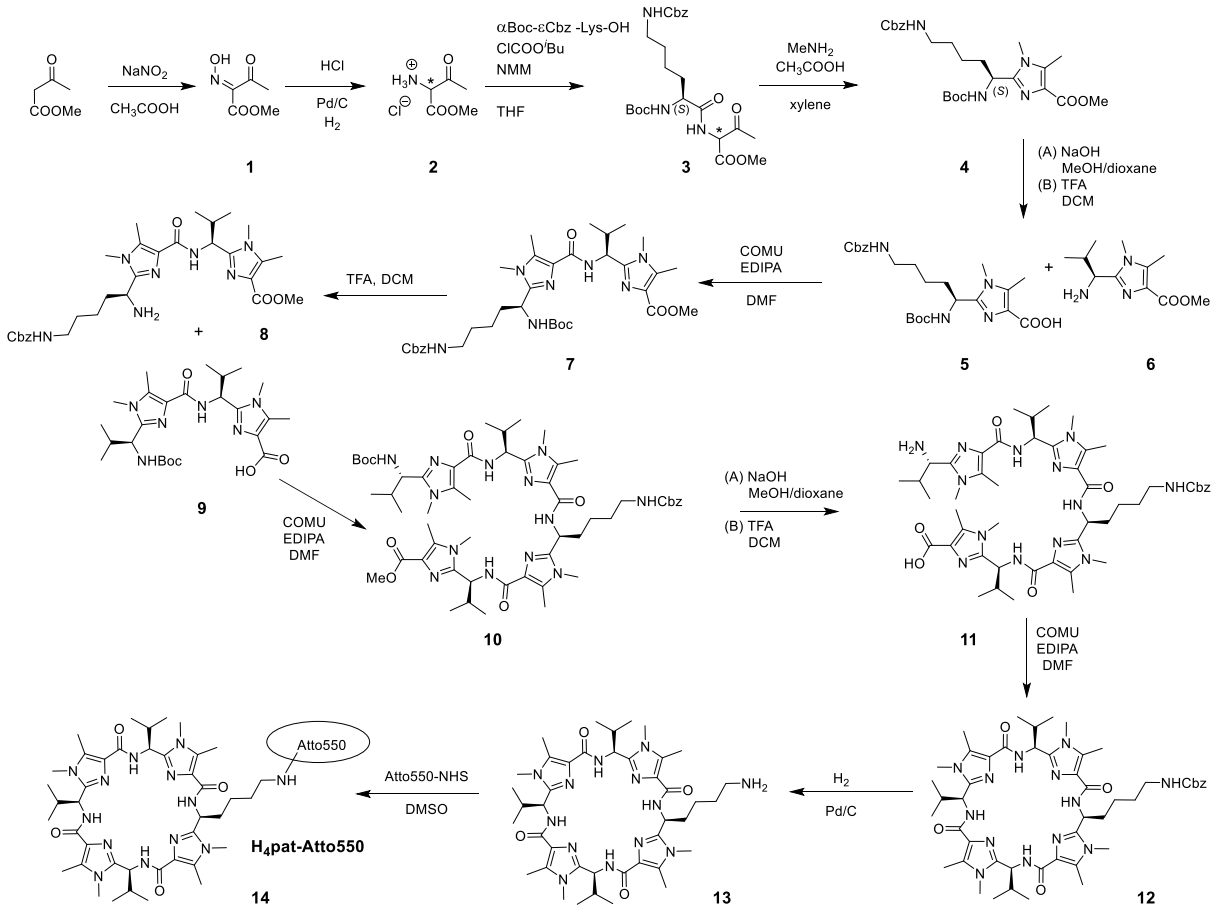
Results

1 Ligand synthesis and structure of the putative copper complexes

The synthesis of the cyclic octapeptide H_4pat-Atto550 was accomplished analogously to the modular approach used for the synthesis of other cyclic peptides prepared for our earlier studies (Scheme 2).[7] The α-Boc ε-Cbz protected lysine was activated by treatment with *iso*butyl-chloroformate and coupled to the keto ester **2** to give the resulting amidoketone **3**, which was reacted with methylamine in the presence of acetic acid under azeotropic removal of water from refluxing xylenes, delivering the monomeric building block **4**. The removal of the Boc protecting group was accomplished by addition of TFA in DCM, which afforded **5**. Analogous to the strategy reported for **4**, the respective valine-imidazole monomer was produced, which afforded after hydrolysis upon addition of NaOH in dioxane/MeOH compound **6**. The coupling of **5** and **6** was accomplished through COMU activation and addition of EDIPA in anhydrous DMF and yielded the *pseudo*-dipeptide **7** in 55 % yield. **7** was subsequently deprotected at the N-terminus, and coupled with **9**, which was obtained analogously to the described route to **7**, yielding the linear *pseudo*-tetrapeptide **10** in 48 % yield. Deprotection of the N- as well as the C-terminus produced **11**, which was then cyclized in a high-dilution reaction to give 27 % of the Cbz-protected cyclic peptide **12**. This was followed by deprotection of the ε-N-terminus upon treatment with H_2 and 5 % palladium on carbon producing **13**, which could then be reacted with the NHS activated ester of the reporter group Atto550. The fluorescence-tagged cyclic *pseudo*-octapeptide H_4pat-Atto550, **14**, was purified by HPLC and characterized by UHPLC-MS as well as by UV/vis spectroscopy.

The choice of the right fluorescent dye was crucial, since *Prochloron* is one of the few prokaryotic oxygenic autotrophs containing chlorophyll *b* in addition to chlorophyll *a*. This means that the range for observation, often referred to as "green gap" between 520 nm and 600 nm, is relatively narrow.[16]

Therefore, as a photophysically sensitive group Atto550 was chosen with an excitation maximum of λ_{exc}=554 nm and an emission maximum of λ_{em}=574 nm.



Scheme 2. Synthesis of H_4pat-Atto550, **14**; the syntheses of **6** and **9** are omitted for clarity and are accomplished analogously as described in [7].

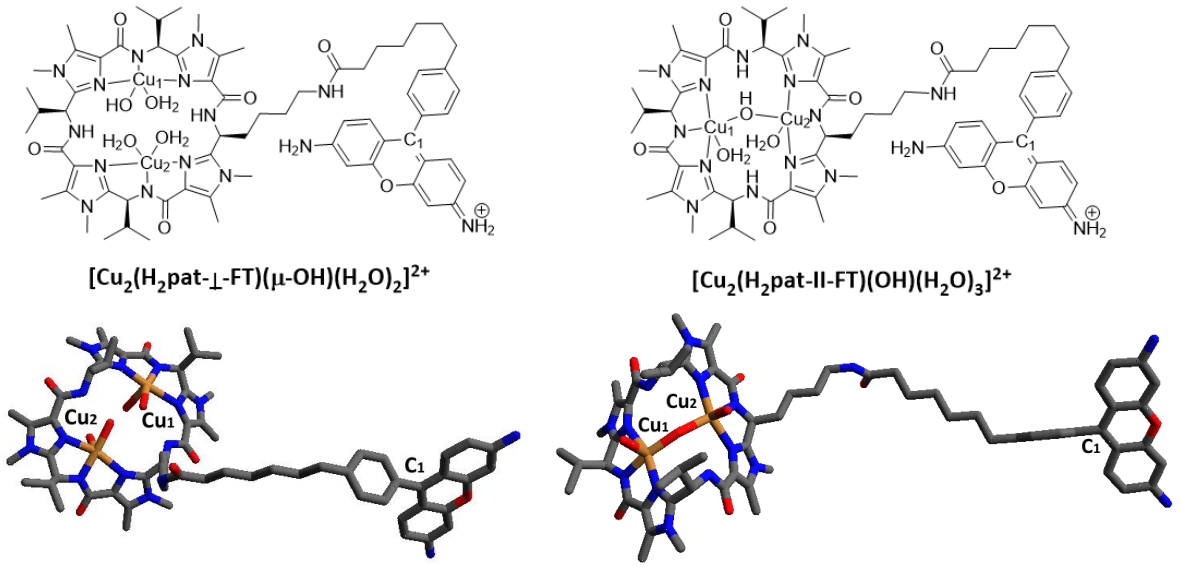


Figure 1. Models of dinuclear [Cu_2H_2pat-FT] complexes; left: Cu^{II} coordinated by three water molecules and a hydroxido ligand, C_4-chain outside the coordination site of the Cu^{II} ions (⊥); right: Cu^{II} coordinated by two water molecules and a bridging hydroxido coligand, C_4-chain inside the coordination site of the Cu^{II} ions (‖), FT=fluorescent tag.

From structure optimization of a conjugate with a rhodamine derivative (the chemical structure of Atto550 is not available) it became clear that the distance between the Cu^{II} centers and the fluorescent tag (C_1, Figure 1) is between 13.5 and 25.4 Å (Cu_1 and Cu_2), depending on where the side chain is located relative to the Cu^{II} binding sites, *i.e.* parallel or perpendicular to the $Cu^{II}\cdots Cu^{II}$ vector. For the perpendicular arrangement of the side chain to the $Cu^{II}\cdots Cu^{II}$ vector distances between 13.5–21.7 Å are expected, whereas the C_1-Cu_n distance is approx. 18.5–25.4 Å for the parallel orientation.

2 Fluorescence and *in vivo* experiments

2.1 Titration

Initially, the sensitivity of the fluorophore-labeled patellamide H_4pat-Atto550 towards Cu^{II} (1.8 µM patellamide-dye conjugate in 100 mM aqueous TRIS buffer, pH=8.2 as observed for sea water)[17] was determined *in vitro* by titration with Cu^{II} (0.125–10 eq). A strong fluorescence quenching was observed upon addition of Cu^{II} (Figure 2).

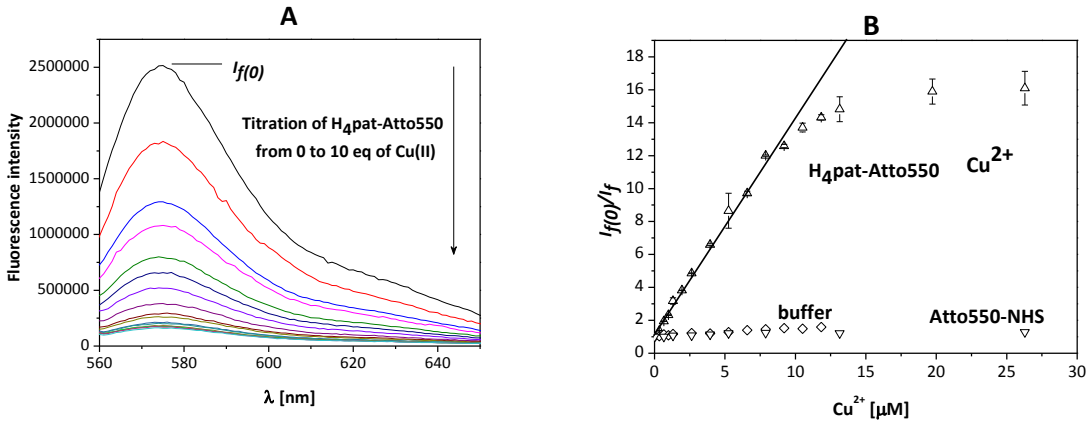


Figure 2. Titration of H_4pat-Atto550 with Cu^{II}, measured by fluorescence spectroscopy (aqueous solution of H_4pat-Atto550 in 100 mM TRIS buffer, pH=8.2, T=293 K; concentrations: [H_4pat-Atto550]=1.8 µM [Atto550-NHS]=2.3 µM). **A** Full emission spectrum and **B** Stern-Volmer plot (H_4pat-Atto550 + Cu^{II}, up triangles) compared to titrations of the conjugate H_4pat-Atto550 + buffer (diamonds) and Atto550-NHS + Cu^{II} (down triangles),

The control experiments, *i.e.* titrations of H_4pat-Atto550 with equivalent volumes of buffer (instead of Cu^{II}) and of Atto550-NHS with Cu^{II}, led to significantly smaller fluorescence intensity decreases (Figure 2B). The quenching behavior of the fluorescence-dye-conjugate is biphasic as the Cu^{II} concentration increases. The initial linear increase in fluorescence quenching gradually levels off at excess Cu^{II}. Static quenching is expected due to Cu^{II} complexation of the patellamide ligand in the starting phase. Fluorescence lifetime measurements, as shown in Figure 3, confirm this assumption as no significant changes of the fluorescence lifetime of Atto550 are observed upon addition of Cu^{II}.

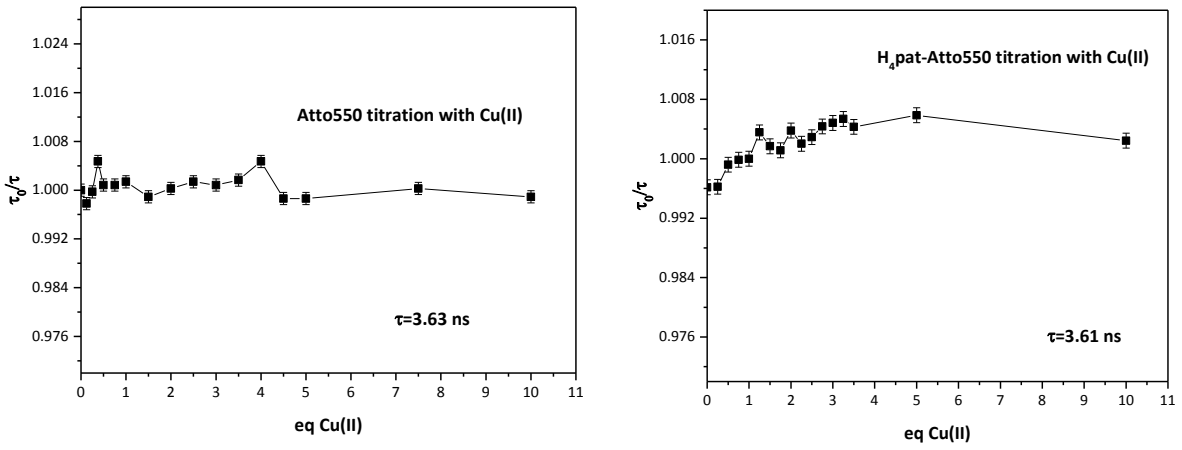


Figure 3. Ratio of fluorescence lifetimes τ_0/τ dependent on the amount of added Cu^{II} (100 mM TRIS buffer, pH=8.2, RT; concentrations: [H_4pat-Atto550]=1.8 µM, [Atto550-NHS]=2.3 µM).

The second phase of the quenching might be due to a saturation effect or could result from the formation of a dinuclear species while the first phase then would be the formation of the corresponding mononuclear complex. However, binding of Cu^{II} to patellamide derivatives has been found to show cooperativity, *i.e.* it is not likely that formation of dinuclear complexes only occurs after addition of 10 eq of Cu^{II}.[2, 9] In first approximation, the model for static quenching is applied to the initial constant slope of the Stern-Volmer plot to the equivalent point (Figure 2B, eqn. 1).[18] Here, I_f is the measured fluorescence intensity at the metal ion concentration of each titration step. A linear fit yields the Stern-Volmer constant K_s of the Cu^{II} patellamide complex.

$$\frac{I_{f0}}{I_f} = 1 + K_s\,[Cu^{II}] \qquad (1)$$

The fit estimates K_s to be 1.35(2) x 10^6 M^{-1}, and this value is very similar to the stability constants for the dinuclear Cu^{II} complexes of the model patellamides (Scheme 1) H_4pat^1 (K_s = 1.7 x 10^6 M^{-1}) and H_4pat^2 (K_s = 4.0 x 10^4 M^{-1}), determined by isothermal titration calorimetry in a methanolic solution.[19] Interestingly, the complex of H_4pat-Atto550 with Cu^{II} seems to be more stable than the related H_4pat^2 complex as both have the same backbone and only differ in one side chain. However, the model used for the determination of the Stern-Vollmer constant does not describe the whole set of experimental data and the conditions used for the different experiments (ITC, fluorescence spectroscopy) are not identical. Therefore, further investigations of the binding of Cu^{II} to H_4pat-Atto550, *e.g.* by ITC, are needed to validate the binding affinity.

2.2 Uptake of H_4pat-Atto550 by *Prochloron*

In order to find the best method to stimulate the uptake of H_4pat-Atto550 by *Prochloron*, different protocols were investigated. As not many reports are published on the introduction of small peptides into prokaryotes, standard techniques for the stimulation of biomolecule uptake, as established for *Escherichia coli,* were applied,[20] *i.e.* heat shock treatment at 42°C for up to 40 s and incubation at room temperature for up to 30 min. After the treatment, the cells were washed twice with buffer, and subsequently, pulsed amplitude-modulated photosynthesis measurements (PAM) were used to ascertain that the *Prochloron* cells were still photosynthetically active. The amount of patellamide taken up by the cells was determined by flow cytometry; here the excitation wavelengths λ_{exc}=535 nm (excitation of H_4pat-Atto550) and λ_{exc}=640 nm (excitation of chlorophyll *b*) were used (Figure 4). The fluorescence of chlorophyll *b* is used as an internal standard. It is apparent that the heat shock treatment (40 s at 42°C) leads to the same ratio of fluorescence at 575nm and 675nm (FL575/FL675, *i.e.* the ratio of fluorescence by H_4pat-Atto550 and chlorophyll *b*, respectively) of about 0.016, as observed for incubation for 30 min at room temperature. This indicates that the transport of patellamides into *Prochloron* occurs by a passive mechanism. The setup of the experiment leads, after dilution with cyanobacteria and buffer, to the final concentration in the sample of 5.8×10^{-8} M for H_4pat-Atto550. With the expected passive mechanism, this concentration consequently is the maximum expected concentration inside the cells; however, this cannot be independently validated and is also dependent on the corresponding equilibrium constant. Since the emission of chlorophyll *b* is very intense, it was also verified that the observed fluorescence intensity does not solely originate from the fluorescence of chlorophyll *b*. The corresponding experimental data from fluorescence spectroscopy and confocal microscopy are shown in Figure 4.

2.3 *In vivo* fluorescence quenching

Since it could be shown that the fluorescence intensity of H_4pat-Atto550 is dependent on the Cu^{II} concentration *in vitro* (see Figure 3) and that the conjugate is taken up by the cells, in a third step the Cu^{II} sensitivity of H_4pat-Atto550 was investigated *in vivo*. Two different techniques were applied, single cell confocal microscopy and flow cytometry (FCM). Both techniques allow the determination of optical parameters of single complete cells instead of giving average values for the whole population. Additionally, flow cytometry is a technique that quantifies various optical parameters of complete cells simultaneously with a high throughput rate. For these reasons it is a quick and reliable technique, especially when compared to the time consuming preparation and data acquisition required for

confocal microscopy experiments.[21] The advantage of confocal microscopy is that it provides information on the spatial organization of each single cell, which is why both techniques were exploited.

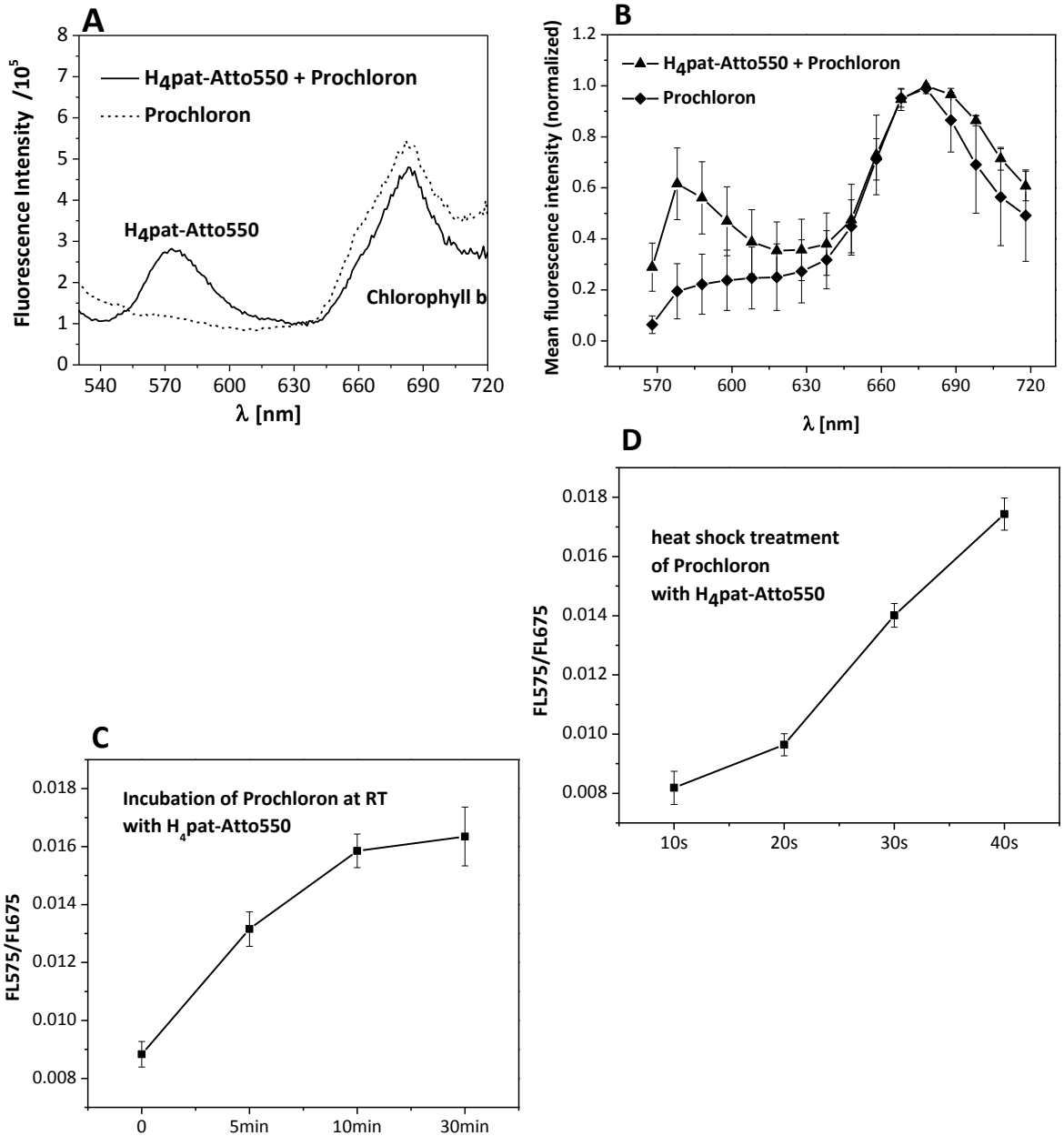


Figure 4. A: Fluorescence intensity (measured with a fluorescence spectrometer) of a *Prochloron* sample treated with H_4pat-Atto550 for 40 s at 42°C (λ_{exc}=500 nm). **B**: Fluorescence intensity from confocal microscopy; *Prochloron* treated with H_4pat-Atto550 for 40 s at 42°C (for raw data see Supporting Information); **C** and **D**: FL575/FL675, *i.e.* ratio of the fluorescence intensity of H_4pat-Atto550 (FL575) to the fluorescence intensity from chlorophyll *b* (FL675).

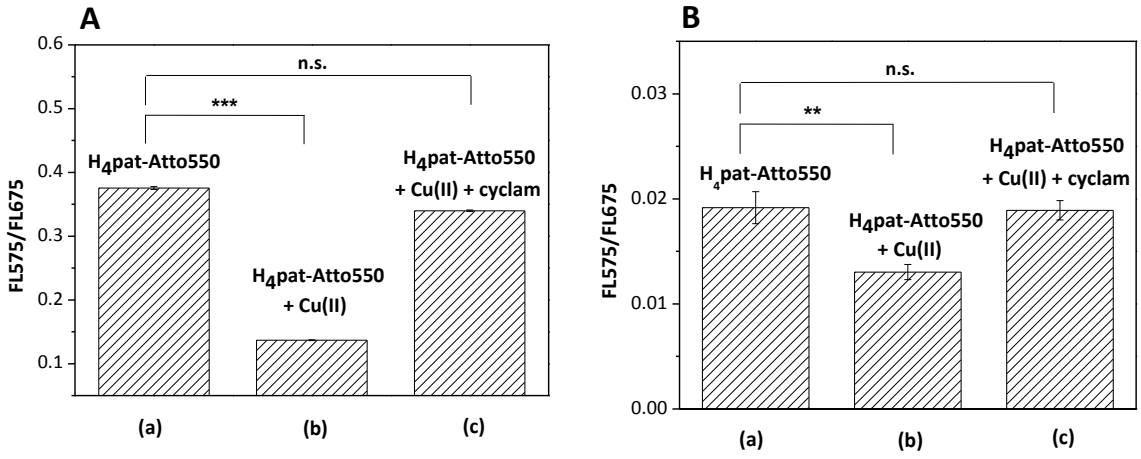


Figure 5. Mean and SEM of FL575/FL675, determined by FCM, for **A** *Prochlorothrix hollandica* (n=12,000) and **B** *Prochloron* (n=4,000), treated (a) with H_4pat-Atto550, (b) with H_4pat-Atto550 and Cu^{II}, and (c) with H_4pat-Atto550 and Cu^{II} and subsequently with cyclam (10 eq) [$p \geq 0.05$ – not significant (n.s.), $p \leq 0.05$ – significant (*), $p \leq 0.01$ – highly significant (**), $p \leq 0.001$ extremely significant (***)].

For the establishment of a valid protocol to investigate the Cu^{II} binding behavior of the fluorescent dye-patellamide conjugate by means of flow cytometry and confocal microscopy, *Prochlorothrix hollandica (P.H.)* was studied. *P.H.* was chosen as it is the taxonomically closest cyanobacterium and readily available from a cyanobacteria database, in contrast to *Prochloron*, which can only be handled for about 1-2 weeks in symbiosis with the host in a fresh water aquarium after sampling from tropical waters. H_4pat-Atto550 was added to the cyanobacteria, which were then heat shocked for 40 s at 42°C and subsequently chilled on ice for 1 min (a). Alternatively, samples (b) and (c) were incubated with H_4pat-Atto550 and 5 eq of a Cu^{II} salt. After rinsing the cells, sample (c) was additionally treated with an excess of cyclam (10 eq, cyclam=1,4,8,11-tetraazacyclotetradecane), applied as a strong competitor for Cu^{II} coordination to reinstall the fluorescence upon removing Cu^{II} from H_4pat-Atto550. All samples were analyzed by FCM, and the mean ratio (and its SEM) of fluorescence observed at 575 nm (corresponding to Atto550) compared to fluorescence at 675 nm (corresponding to chlorophyll *b*, used here as an internal standard) is plotted in Figure 5. For *Prochlorothrix hollandica* samples (a) and (c) are not significantly different, whereas sample (b) shows a large ($p<0.001$) difference compared to (a) and (c). This lends strong support for the hypothesis that Cu^{II} is binding to H_4pat-Atto550 in *Prochlorothrix hollandica*. In addition, the binding constant of the Cu^{II} patellamide complex seems to

be significantly smaller than that of the corresponding Cu^{II}-cyclam complex, which is in accordance with expectations, as the stability constant for $[Cu^{II}(cyclam)]^{2+}$ is log*K* = 14.7 (pH 8.2),[22] orders of magnitude higher than what was observed from photophysical measurements for the H_4pat-Atto550 ligand studied here (*vide supra*). Consequently, the established protocol was also applied to *Prochloron* cells. The results are summarized in Figure 5B. It emerges that sample (a) (0.01874 ± 0.0015) and (c) (0.02026 ± 0.0010) are not significantly different, but sample (b) is significantly different from (a) and (c), exactly as observed for *Prochlorothrix hollandica*. This implies that a Cu^{II}-patellamide complex is stable in the *Prochloron* cells and could possibly act as a catalyst, as observed *in vitro*. It should be noted here that in spite of *Prochlorothrix hollandica* being the taxonomically closest cyanobacterium it contains significantly less chlorophyll *b* than *Prochloron*, which leads to larger values for the observed ratios FL575/FL675 in the range of 0.2-0.4 compared to the values observed for *Prochloron* (0.013-0.020).

Samples prepared identically as described for FCM were also investigated concerning their relative brightness at 574 nm by confocal microscopy, and the results are depicted in Figure 6. In order to validate that the observed brightness originates solely from the fluorescent-dye-patellamide conjugate, the relative brightness of *Prochloron* at λ_{em}=574 nm was plotted as the first bar in Figure 6.

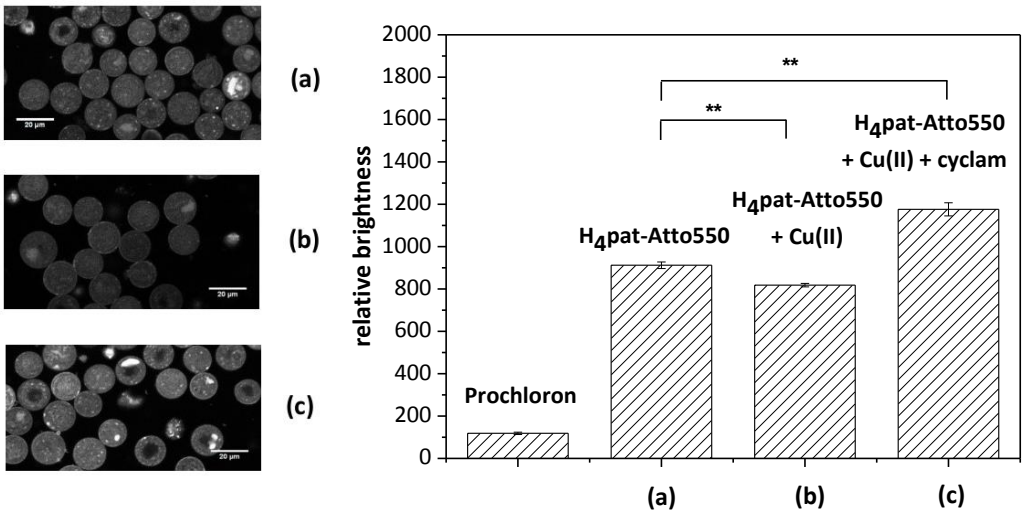


Figure 6. Left: Confocal microscopy pictures of *Prochloron* cells and right: mean and SEM of FL574 for *Prochloron* (n=600) treated (a) with H_4pat-Atto550, (b) with H_4pat-Atto550 and Cu^{II}, and (c) with H_4pat-Atto550 and Cu^{II} and subsequently with cyclam.

Sample (b) shows a significantly lower brightness compared to samples (a) and (c) but at the same time sample (c) is significantly brighter than sample (a). This implies that Cu^{II} is binding to the patellamide-fluorescent-dye conjugate. Upon addition of an excess of cyclam the Cu^{II} that was added as well as endogenous Cu^{II} ions are removed by cyclam. Although FCM does not show a significant difference for samples (a) and (c) of *Prochloron* (Figure 5), it is worth noting that the mean of (c) is higher than the

mean observed for (a) although the difference is not significant. Moreover, the sample size for FCM is ~12,000 per population, whereas the populations investigated with confocal microscopy consist of ~600 *Prochloron* cells. Importantly, unlike the FCM data (normalized to background chlorophyll), the data from confocal microscopy are not normalized.

To exclude the occurrence of an 'inner filter effect', UV-vis-NIR absorption measurements of a sample of *Prochlorothrix hollandica* lysate, treated identically (same population density and Cu^{II} concentration) to the samples prepared for FCM and confocal microscopy, were carried out (see Figure 7). These experiments were aimed to elucidate whether putative Cu^{II} complexes with intracellularly abundant chelators like amino acids absorb strongly at 570-620 nm, causing the fluorescence intensity of Atto550 to appear attenuated.

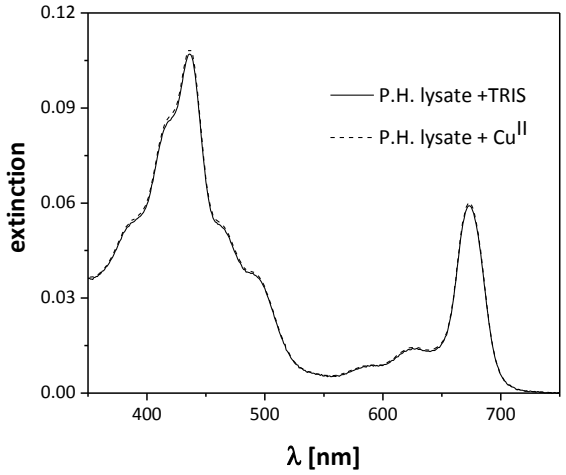


Figure 7. Absorption spectrum of a *Prochlorothrix hollandica* lysate solution treated with Cu^{II}, $[Cu^{II}]=2.9x10^{-7}$ M, and a solution of *P.H.* lysate solution treated with the equivalent volume of TRIS buffer.

From these measurements it appears that the treatment of the cells with Cu^{II} does not lead to any change in the absorption spectrum of the corresponding cell lysate. Consequently, the potential formation of Cu^{II} complexes with abundant chelators does not alter the absorption behavior of the cells significantly.

2.4 Copper ions in living cells

Knowledge of the concentration of copper ions in living cells and their oxidation state is of significance for *in vivo* studies and, unfortunately, this is not well studied for cyanobacteria. It is known that mammalian (eukaryotic) cells exhibit a reductive interior condition, and the free copper cations are thus present as Cu^{I}.[23] The copper ion homeostasis is regulated by a copper ion importing and exporting system and leads to a copper ion concentration in the cell in the femtomolar range.[23] Similar transport systems were also found in the prokaryotes *Enterobacteria*.[24] However, here, interior copper ion

concentrations in the micromolar range were reported, as compared to the nanomolar range (10^{-8} M) in the surrounding medium.[24] From a proteomic study of the cyanobacterium *Synechocystis sp.* it has emerged that the export mechanism is the main regulator of the copper cation concentration.[25] However, exact copper cation concentrations for cyanobacterial cells are not published so far. In 2001 it was reported that after acidic lysis of *L. patella* with *Prochloron* cells, the Cu^{II} concentration in the whole organism corresponded to a 10^4 times enrichment compared to the surrounding sea water.[26,5] From these results a maximum intracellular concentration of 1.6 µM can be estimated for *L. patella* with *Prochloron* (including the copper cations that are potentially bound to storage proteins).[5]

The question about the reductive/oxidative milieu in the cells remains unanswered. It is known that, depending on the level of irradiance, the pH is fluctuating and the same is true for the level of oxygen saturation.[17, 27] As a consequence of the oscillation during day/night cycles, the oxidative environment in the cells is expected to be correlated to the level of oxygen in the cells. If this is the case, one can expect an alkaline environment (pH 11) as well as dioxygen super saturation upon solar irradiation[17] and consequently copper cations to be present as Cu^{II}. In the dark, on the other hand, an interior milieu of pH 7.0, in combination with an anoxic environment is expected,[17] which would favor Cu^{I}. To date, the coordination chemistry of Cu^{I} to patellamides has not been studied in detail. Research in this field would improve the understanding of relative stabilities of the putative Cu^{I} *versus* Cu^{II} complexes. This leaves us to propose that the maximum internal concentration of 1.6 µM for Cu^{II} and 18 µM for Zn^{II} is expected in *L. patella* with *Prochloron* and that copper is possibly divalent during exposure to sunlight and monovalent during darkness.

Furthermore, knowledge about potential chelators other than the patellamides, in particular their concentrations and binding affinities for Cu^{I}/Cu^{II} and Zn^{II} in the cells, is crucial for this study since most likely a major part of the intracellular copper and zinc cations are complexed, leading to a much smaller concentration of uncoordinated metal ions (probably as aqua complexes). Again, no data for *Prochloron* or similar cyanobacteria (prochlorophyta) are available. Absolute metabolite concentrations for *E. coli* have been published,[28] and indicated that the concentrations of amino acids like glutamate, glutamine, aspartate, valine and alanine, to mention but a few, are in the millimolar range.[28] Apart from amino acids, other metabolites like ATP, GTP and citrate are also present in similar concentrations.[28] In a study on the amino acid composition of the dried cyanobacteria *Nostoc spongiaeforme* and *Rivularia bullata*, all peptides in the cell were hydrolyzed for sample preparation, and the amount of amino acids reported does therefore not correspond to the concentration of amino acids in solution. If one nevertheless uses these values and expects the native cyanobacteria to have an approximate water content of 70 %,[29] cytosol concentrations of the amino acids glycine, threonine and leucine of 37.9 mM, 25.5 mM and 28.8 mM can be anticipated, and this resembles the composition of all proteins and metabolites in cyanobacteria. The binding constants of 1:1 amino acid

Cu^{II} complexes are in the range of log$K$=7-9, and for 2:1 complexes, one expects logβ values between 12 and 16.[30] These stability constants are significantly higher than those for patellamide Cu^{II} complexes, typically in the range of logK_1K_2=4-6, depending on the patellamide backbone.[2] Despite the lack of exact knowledge of the amino acid concentrations in cyanobacteria and *Prochloron* in particular, one can assume from the stability constants that the formation of Cu^{II} amino acid complexes should be preferred over the formation of patellamide complexes. However, as mentioned earlier, the stability constants greatly depend on the pH, the oxidation state of the copper cation and the relevant local concentrations. In addition, so far no experiments have been carried out to elucidate whether or not the Cu^{II} patellamide complexes might be integrated in a protein as a prosthetic group. That would not be unexpected, since in nature the majority of metal complexes are embedded within the active site of proteins.[31-34] If that was the case, the stability constants measured previously for patellamides would be challenged.[2]

Conclusion

In conclusion, the synthesis of the fluorescent-dye-derivatized patellamide H_4pat-Atto550 is reported together with optical spectroscopy and photophysical studies related to its Cu^{II} ion sensing. The fluorescence of the ligand is Cu^{II} concentration dependent with a Stern-Volmer constant of K_s = 1.35 x 10^6 M^{-1}, which is of the same order of magnitude as the stability constants of similar model patellamides.[2] It is shown that the model peptide H_4pat-Atto550 is taken up by *Prochloron* cells, probably by a passive mechanism. The imaging *via* flow cytometry and confocal microscopy implies that a Cu^{II}-patellamide complex forms in the *Prochloron* cells, as observed *in vitro*. The formation of Cu^{II} complexes inside *Prochloron* cells indicates that the catalysis of various hydrolysis reactions observed *in vitro*, carbonic anhydrase, phosphatase, lactamase and glycosidase,[1, 11, 12] and possibly others not yet explored, might be the metabolic purpose of the patellamides. The corresponding Zn^{II} complexes, which have also been shown to form and to be catalytically active are less likely to be of biological relevance,[3] and the Cu^{II} complexes might therefore be a rare case of Cu^{II} hydrolase enzymes. So far, it is not clear, whether mono- or dinuclear complexes are formed *in vivo*, and their structures are unknown. Structural information of the *in vitro* Cu^{II}-patellamide complexes was obtained by EPR spectroscopy.[2, 8, 9] Therefore, other reporter groups, spectroscopic techniques and mechanistic experiments are needed to answer still open questions of these interesting systems.

Experimental Section

Materials and Methods

Some of the reactions reported were carried out under an inert atmosphere of argon or nitrogen using standard Schlenk-techniques. Glassware was heated and dried under vacuum prior to use. All chemicals were purchased from Sigma-Aldrich GmbH, ABCR GmbH & Co. KG and Merck at the highest available purity. Dry solvents were purchased and used as delivered. For optimized comprehensibility, names of compounds synthesized were simplified instead of usage of the exact IUPAC name.

High resolution mass spectra (HR-ESI MS) were recorded on a Finnigan MAT8230 and Joel JMS-700 spectrometer by Dr. Jürgen Gross and co-workers at the mass spectrometry facility in the Organic Chemistry department of Heidelberg University on a ICR Apex-Qe and a JEOL JMS-700. Atmospheric pressure chemical ionization mass spectra (APCI MS) were recorded on a Waters UPLC-SQD2 single quadrupole.

Nuclear magnetic resonance spectra were recorded on a Bruker Avance III (15.1 T; 600 MHz for ^{1}H) spectrometer equipped with a cryoprobe. Chemical shifts δ are given in ppm and coupling constants J in Hz. All spectra were calibrated using the residual ^{1}H- or ^{13}C-signals of the deuterated solvents. Spectra were recorded at 298 K.

The following abbreviations were used to describe the multiplicities of the signals: s (singlet), d (doublet), t (triplet), qn (quintet), m (multiplet). Signals were assigned using DEPT, HSQC and HMBC spectra.

The purification of the dye conjugate H_4pat-Atto550 was performed with the HPLC (High Performance Liquid Chromatography, Agilent, Waldbronn, serial number 1100), equipped with a binary pump G1312A, a degasser G1322A, a diode array detector G1315 A and a fluorescence detector G1321A. The chromatographic separation was performed using the reversed phase column from Knauer, Berlin (250 mm, 4 mm inner diameter, ODS-Hypersil with pore diameter of 5 µm).

Statistical Analysis

A One-way ANOVA was used for statistical analysis. The required level of significance was defined to be 5 % ($p \geq 0.05$ – not significant (n.s.), $p \leq 0.05$ – significant (*), $p \leq 0.01$ – highly significant (**), $p \leq 0.001$ extremely significant (***)).

Syntheses

The synthesis of H_4pat-Atto550 was accomplished following a method analogous to the previously reported *pseudo*-peptide syntheses and is described in detail in the Supporting Information.

UV-vis and Fluorescence Spectra

As a copper(II) salt $CuSO_4x6\ H_2O$ was used for all photophysical experiments and fluorescence titrations.

UV-vis absorption spectra were recorded with the Cary 60 UV-vis spectrometer (Agilent) at room temperature and fluorescence spectra (quenching experiments) of the H_4pat-Atto-550 - monitored with the 'Fluorolog 3' fluorescence spectrometer (Horiba). The measurements were carried out in quartz glass cuvettes. Fluorescence spectra (quenching experiments) of Atto550-NHS were recorded with 'Cary Eclipse 500' fluorescence spectrometer (Varian, Darmstadt) with temperature control. The measurements were carried out in dark quartz glass cuvettes (Suprasil©, Hellma, Müllheim).

Fluorescence lifetime measurements: Titration of H_4pat-Atto550 with Cu(II)

Fluorescence lifetimes were determined on a 'Fluotime 100' spectrometer with time-correlated single photon count card 'TCSPC TimeHarp 200' from PicoQuant. The excitation wavelength was generated by a pulsed diode 'PLS 500' (500 nm, 40 MHz) from PicoQuant with a pulse width of 300 ps tuneable fibre coupled diode. The instrument response function was measured with colloidal SiO_2 nano-particles (LUDOX®HS-40 (420816), Sigma-Aldrich). During the measurement, the excitation light was filtered with suited high-pass filters, data were analysed with the programme 'FluoFit' from PicoQuant.

Fluorescence quenching experiments

The metal salt used (analytical grade, $CuSO_4$x6 H_2O 0.16 M aq. solution) was diluted in doubly distilled H_2O (0.01 M); a solution of H_4pat-Atto550 was adjusted to 1.8 µM and prepared from high concentration aqueous solutions (doubly distilled water) by dilution into 100 mM TRIS buffer, pH 8.2, doubly distilled H_2O.

To 200 µL of H_4pat-Atto550 in the required concentration, the metal salt in the required concentration was added in steps of 1 µL before measuring the fluorescence spectrum. The intensities were corrected by the corresponding dilution factor; the reported data are averages of triplicates.

Cyanobacteria handling, Field Site and Sample Collection

For the experiments *Prochlorothrix hollandica* (SAG 10.89) and *Synechococcus leopolienses* (SAG 1402-1) were purchased from the Culture Collection of Algae at Göttingen University and stored on the bench at room temperature and without direct light irradiation for a maximum of two weeks.

For *Prochloron* fom *L. patella*, individual patches and their coral rubble substrate were collected with a hammer and chisel. Intact specimens (5-25 cm^2) of 5-20 mm thick *L. patella* were collected at low tide on snorkel on the outer reef flat and crest off Heron Island (First Point: S23°25’49.8, E151°57’15.8, Blue Pools: S23°26’03.2, E151°55’18.1,) in May 2016 covering coral patches on the outer reef flat and down to ~4 m depth on the reef crest. All samples were immediately transported in a bucket with sea water back to Heron Island Research Station and subsequently kept in an outdoor aquarium, which was continuously flushed with fresh aerated sea water (24–26°C) from the reef. As reported

previously,[17] it is necessary to keep a continuous strong mixing of water in the aquarium to avoid the degradation of the ascidians. The aquaria were covered by a shading cloth, to prevent excessive solar irradiance.

Samples were then transported to the University of Queensland, St Lucia Campus, in a plastic bag, filled with fresh aerated sea water and oxygen, and stored in a cold box at 25°C by plane and 6 h later put in an aquarium with synthetic sea water and constant water flow. The seawater is generated from Tropic Marine salt[35] mixed with water (that was purified *via* reverse osmosis) to a salinity of 35 ppt (23-25°C; the water is not sterilized but pumped through system of sediment and biological filters). Under these conditions we were able to keep the sampled ascidians healthy and with *Prochloron* actively photosynthesising *for nine days.*

Prochloron was collected using a pipette and by gently squeezing the middle section containing the cloacal cavity harbouring the deep green symbiont.

Prior to all measurements the viability of the cells was tested with a ToxyPAM.[36-38] Generally, specimens showed a high maximum PSII quantum yield of >0.6 for several hours after such handling indicative of fast recovery and minimal stress on *Prochloron* (Heinz Walz GmbH, Effeltrich).

1 cm x 1 cm patches were additionally cut from *L. patella* with a sterilized razor blade and stored in 20% glycerol solution at –80°C, transported back to St Lucia on dry ice and stored at –80°C. Healing was observed for the patches which had been cut.

Protocol for the treatment of *Prochloron* (preparation of FCM and confocal microscopy experiments)

For the preparation of samples for FCM and confocal microscopy 40 µL of a 2.4×10^{-6} M solution (100 mM TRIS buffer, pH 8.2) of H_4pat-Atto550 were added to 30 µL of the respective cyanobacteria at OD_{750}=0.8 in BG 11 medium (sample a). For sample b and c 40 µL of a 2.4×10^{-6} M H_4pat-Atto550, with 5 eq of copper(II) ($CuSO_4 x 6H_2O$) were used for the treatment of the cells. After heat shock treatment for 45 s at 40°C the samples were put on ice for 1 min. Washing with 50 µL buffer followed. The cells were then resuspended in 10 µL of buffer and 90 µL of a 4 % PFA solution was added the cells were incubated for 15 min. Again the cells were washed with 50 µL buffer and either (samples a and b) resuspended in 50 µL of TRIS or (c) treated with 10 eq of cyclam for 10 min. Sample c was after that also washed with 50 µL of buffer and resuspended in 50 µL of TRIS. All experiments were carried out in triplicates. For FCM the final suspension were analyzed within 1 h after preparation.

For confocal microscopy the samples were placed in 8-well glass slides (ibidi GmbH, Lab-Tek) and the solvent was slowly evaporated under reduced pressure in a desicator (1 h) and the cells were analyzed immediately.

Flow cytometry (FCM)

The flow cytometry experiments were performed on a C6 cytometer (Accuri). Fluorescence excitation was at λ_{exc}=520 nm and λ_{exc}=640 nm, and the respective emission was detected using a 585/42 nm (FL1) and a 694/40 nm (FL2) filter. The mean ratio (and the respective SEM) of the fluorescence observed at FL1 compared to FL2 is plotted. All flow cytometry measurements include minimum 4,000 events in the gated area and were carried out in triplicates.

Confocal Microscopy

All *Prochlorothrix hollandica* and *Synechococcus leopoliensis* samples were measured using a confocal laser scanning microscope (Leica SP5), applying 63x water immersion objective. The image parameters were chosen 2048x2048 by 16 bit at 200 Hz. Each picture represented an area of 246x246 µm, representing a two-fold oversampling by a spatial resolution of 132 nm per pixel. Atto550 was excited *via* pulsed whitelight laser (80 MHz, SuperK Extreme, Koheras) at 561 nm, intensity 70 %. Emission detection was done using photomultiplier tubes (PMT) within 574-680 nm. At least 10 measurements at random positions within one Lab-Tek chamber were conducted per condition.

Prochloron samples were measured using a confocal laser scanning microscope (ZEISS LSM 710) applying 63x oil immersion objective (C-Apochromat 63x/1.2 W Korr). The image parameters were chosen 1024x1024 by 16 bit at 100 Hz. Each picture represented an area of 135x135 µm, representing a two-fold oversampling by a spatial resolution of 132 nm per pixel. Atto550 was excited *via* pulsed white light laser at 561 nm, intensity 70 % (561 nm continuous wave Diode-Pumped Solid State Laser DPSS on a Zeiss LSM 710 instrument). Emission detection was done using photomultiplier tubes (PMT) within 556-606 nm and 624-735 nm. At least 10 measurements at random positions within one Lab-Tek chamber were conducted per condition.

For quantification, Fiji (http://fiji.sc/) software was used. Images were filtered by median, followed by background subtraction. Cells were selected using Otsu thresholding and subsequent particle analysis, followed by calculation of median intensity and standard error.

Absorption measurement of the *Prochlorothrix hollandica* lysate

5 mL of *Prochlorothrix hollandica* (*P.H.*) from culture were centrifuged down at 18.000 g for 5 min. The supernatant was discarded, and cells were resuspended to OD_{600}=0.8 in TRIS buffer, pH 8.2. Disintegration was done using a sonicator (BW2070, Bandelin electronics Berlin GmbH) for 5x10 s at 75% of maximum power. In between repeats, lysate was chilled on ice for 1 min. Cell debris was spun down for 15 min at maximum speed. The supernatant was measured for absorbance properties (Cary 60 UV-vis spectrometer (Agilent) at room temperature) with or without $CuSO_4$ (final concentration as

described in the experimental section (FCM and confocal microscopy): 2.9 x 10^{-7} M). As control the same volume of TRIS buffer was supplemented.

Supporting Information

The Supporting Information covers details on the synthesis of H_4pat-Atto550, fluorescence lifetime curves for the H_4pat-Atto550 conjugate, as well as raw confocal microscopy pictures on the uptake of H_4pat-Atto550 by *Prochloron*. In addition, the results from the investigation of *Synechococcus leopoliensis* and *Prochlorothrix hollandica* by FCM and confocal microscopy are given. Coordinates of the computed structures and details on the computations are also presented in the Supporting Information.

Acknowledgements

For financial support we are grateful to the German Science Foundation (DFG, HGS MathComp), ARC and the University of Heidelberg. We thank Sandra König for support with flow cytometry, as well as Prof. Peter Vize and Daniel Wuitchik for help with the collection of *L. patella* samples on SCUBA at Heron Reef. We are grateful to Prof. Dr. Thomas Söllner for access to his labs, and to Prof. Dr. Jürgen Müller's and Jake O'Brien's efforts to allow AE to use their ToxyPAM. The excellent technical support by the staff at Heron Island Research Station, University of Queensland, as well as the support from Dr. Steven Mason, Prof. Bernhard Degnan and Dr. Jabin R. Watson is acknowledged. We are grateful for the computational resources provided by the bwForCluster JUSTUS, funded by the Ministry of Science, Research and Arts and the Universities of the State of Baden-Württemberg, Germany within the framework program bwHPC-C5. GS acknowledges the Australian Research Council for support in form of a Future Fellowship (FT120100694).

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TOC

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Ihre Zeichen, Ihre Nachricht vom	Unser Zeichen, unsere Nachricht vom	Telefon, Name	Datum
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Dear Editor

We were not aware that this publication is considered as a revision of our original communication sent to Angewandte Chemie. Anyway, we very carefully analyzed the original reviews and they helped us to rewrite the manuscript as a full paper and to significantly improve it. Much of the text is new, specifically the parts where we discuss the amount of free copper and its redox state in cells. The manuscript also contains some new results due to experiments done on request by the reviewers. Attached below is a point by point answer to the original reviews.

Best regards Peter Comba

Reviews and answers (in blue) of the original Angew. submission

Reviewer 1: Comba et al. report on the Cu(II) coordination to patellamides inside Prochloron cells. The work is very interesting but also extremely specialised.
Thank you for the positive comment. We agree that *L. patella* are special creatures but the more general question is, how copper coordination chemistry with the natural macrocycles can be performed in living cells – the corresponding in-vitro chemistry was published, to some extent in Chem.Eur.J.

Dinuclear Cu(II) patellamide complexes are relevant for biological hydrolysis reactions which are rare for copper metalloproteins. Pattelamides are octapeptides and the authors show a multistep synthesis to a fluorescence markered octapeptide. The design of the study is very interesting: synthesis of the octapeptide, linking to a tag, study of the copper complexes and quenching of the fluorescence by Cu(II). By DFT, they study how far the fluorescent tag is dangling from the copper ions.
We agree with the reviewer that the in-vitro chemistry shows that these complexes are the most efficient models for carboanhydrase and if this would be the natural role, these would be the first Cu-based carboanhydrases. This is the beauty and novelty of the project and we therefore think that this is interesting for a general audience like Chem.Eur.J.

By studying this model and the true patellamides by fluorescence spectroscopy (Stern-Volmer plot) they reveal the binding of copper in the biological system. The study is well performed but sometimes hard to understand (e.g.

biochemical jargon: p.5 in hospite). The interference of the fluorescence with chlorophyll b and its relevance for the presented study is not well explained. As result they show that the model peptide is taken up by Prochloron cells. Unfortunately, the conclusion is a bit wavy and unclear. Here, the relevance to the broader audience does not come clear. Perhaps, it is more suited for ChemBioChem as Full Paper.
As most of the paper was rewritten, we have taken care of linguistic problems and clearity of the text. Since we did not have space restrictions, we have taken care to fully explain important details. We also hope that the new section on copper in cells helps to fully appreciate the conclusions: Cu(II) is coordinated to the ligand, the answer to the title question unambiguously is “yes”.

Reviewer 2: Patellamides have fascinated biologists and chemists alike for decades, and their biological function remain elusive until today. The ability of patellamides to coordinate metal ions, including Cu(II) and Zn(II) is well established and supported by several important contributions of the Comba group; however, it remains unclear whether such complexes are of biological significance. The manuscript addresses this central and equally challenging question by a combination of in vitro and in vivo studies. Although the presented flow-cytometry and microscopy data appear to support the notion that the Cu(II)-complexes of patellamides are formed in vivo, it remains unclear to what extent an intracellular pool of Cu(II) is present within a reducing intracellular environment. Even if a sizable pool of Cu(II) would be available for this purpose, there are a multitude of other biological ligands that would coordinate to Cu(II) with higher affinity. For example, cells must maintain a minimum concentrations of free amino acids in order to sustain protein synthesis. Although the absolute concentrations may vary somewhat from organism to organism, the combined concentration of free amino acids likely resides in the millimolar range in most cells. Taking glycine as a representative amino acid, which has a pKa of 9.6 and binds Cu(II) with log betas 8.2 (1:1 complex) and 15.1 (1:2 complex), the estimated buffered Cu(II) concentration for a solution containing 1 mM of glycine and 100 uM of Cu(II) would be around 8 nM (0.1 M ionic strength, 278 K). At the same time, the Cu(II) titration experiments, which were conducted at pH 8.2, suggest a binding affinity of around 10^6 for the formation of a 1:1 complex with H4pat-Atto550, the fluorescent dye conjugate of a model patellamide synthesized for the current studies. Given the high pKa of the amide donor, which is deprotonated upon Cu(II) coordination, the apparent affinity would be one order of magnitude lower at neutral intracellular pH. In light of the measured weak micromolar Cu(II) affinity, formation of [Cu(II)H3pat](+) would not be able to compete with Cu(II) coordination to intracellular amino acids and its concentration in the cytosol is expected to be negligible. The observed Cu(II)-dependence in the flow cytometry and microscopy experiments might be due to inner filter effects as most Cu(II)-complexes absorb in the visible range, thus attenuating detectable Atto550 emission.
We are grateful to the reviewer for these critical remarks and these have now been addressed, primarily in Section 2.4 Copper ions in living cells. We have also made experiments showing that there is no inner filter effect (see remark ahead). Our conclusion therefore is that Cu(II) is coordinated to H4pat-atto550, and this is discussed in detail.

It should be also noted that the molar extinction coefficient of Atto550 is very low at the chosen excitation wavelength of 488 nm, thus greatly attenuating the brightness of the fluorophore and making the emission signal more susceptible to interferences. Although excitation at the peak absorption

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of Atto550 at 550 nm would produce a much brighter signal, it would likely interfere with the chlorophyll emission. Given this apparent spectral overlap, it is unclear why the authors did not synthesize a fluorescent dye conjugate with a better matching excitation maximum at 488 nm. No information has been provided regarding the total intracellular copper concentration prior and after Cu(II) supplementation, and it remains unclear what effect would be expected based on such data.
see above

In summary, concluding from simple thermodynamic considerations and the presented data the claims outlined in this manuscript are difficult to defend. Nevertheless, the synthesis of H4pat-Atto550 together with the Cu(II) coordination studies would be certainly worthwhile publishing in a more specialized journal.
We appreciate that the reviewer likes the synthesis of our patellamide-fluorescence probe. This is now described in the main paper. However, we also believe that the experimental results show convincingly that Cu(II) is coordinated to the ligand in the cells.

Some additional comments for consideration:

Page 1, #42: Although Scheme 2 outlines the synthesis of compound 13 in great detail, the discussion in the main text is limited to a single sentence. As the synthesis is clearly not the main focus of the manuscript, it would be sufficient to refer to the synthetic scheme in the Supporting Information.
Details are now given in the text since this is a full paper.

Page 3, #9: Why were the Cu(II) titrations conducted at pH 8? At such high pH, excess Cu(II) would be expected to precipitate as Cu(OH)2 (Ksp ~ 2 x 10^(-20)). To avoid hydrolysis reactions, both of the aqua complex and H4pat-Atto550 bound to Cu(II), a slightly acidic pH should be chosen. The affinity at pH 7 could be estimated based on the corresponding pKa's.
pH 8 is relevant in sea water, and the pH in *L.patella / Prochloron* varies as a function of light.

Page 3, #14: what is meant with "titration of H4pat-Atto550 with buffer"?
changed

Page 3, #17: "the initial linear increase in intensity" is an incorrect statement as the intensity decreases (only the quenching ratio I0/I increases).
changed

Page 3, #33: The initial slope of the Stern-Volmer plot was used to extract Ks for the Cu(II)-H4pat-Atto550 interaction, corresponding to a 1:1 complexation stoichiometry. It is unclear how this value can be numerically compared to a dinuclear complex stability constant which would correspond to the product of the stepwise 1:1 and 2:1 equilibrium constants (K1xK2). According to Figure 2B, coordination of a 2nd equivalent of Cu(II) appears much weaker. As stated above, complexation equilibrium likely involves deprotonation of the two amide nitrogens, both of which would be expected to have a rather basic pKa. The intracellular pH would be expected to be one order of magnitude lower than the chosen experiemtnal conditions, which in turn would reduce the derived 1:1 stability constant by 1 logarithmic unit (or two units in the case of a binuclear complex stability constant).
The issue of complex stabilities is now discussed in more detail. Two facts are important: (i) There is cooperativity, i.e. the second copper is bound more strongly than the first. (ii) The conditions are different (H2O vs.

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MeOH/H2O. What we discuss is just orders of magnitude, i.e. the Stern-Vollmer constant is interestingly of the same order of magnitude as K1xK2.

As the authors state, it is indeed surprising that the stability constant of H4pat is almost two orders of magnitude lower compared to H4pat-Atto550, especially since the Atto550 moiety is cationic and thus would be expected to further reduce rather than increase the interaction with Cu(II). Was the lower affinity determined at the same pH?
see above

Page 3, caption Figure 2: The concentration of H4pat-Atto550 should be stated. Which Cu(II) salt was used?
done

Page 4, #10: the cited stability constant for the Cu(II) cyclam complex refers to an amphiphilic derivative, not cyclam itself. As the cyclam pKa's are 11.4 and 10.3, the stability constant should be adjusted to the apparent affinity at pH 8.2.
changed

Reference 16 has been submitted for publication, but is currently not available for review. This material should be provided to the reviewers as well.
now published

Minor corrections:

Carboanhydrase should say "carbonic anhydrase"
Exemplied should say exemplified
Chlorophyll a: "a" should be italicized
changed

Supporting information:

Page 16: Experimental details for the fluorescence lifetime measurements are missing (instrument, excitation source, excitation and emission wavelength, temperature).At least two representative decay profiles (with and without Cu(II)) should be provided. Why is the standard deviation for tau0/tau so large?
added

Page 17: Figure S2 appears to be identical with Figure 2 of the main text. There is no need to reproduce the same figure again.
changed

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